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Interrogating Genes That Mediate *Chlamydia trachomatis* Survival in Cell Culture Using Conditional Mutants and Recombination

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ABSTRACT

Intracellular bacterial pathogens in the family *Chlamydiaceae* are causes of human blindness, sexually transmitted disease, and pneumonia. Genetic dissection of the mechanisms of chlamydial pathogenicity has been hindered by multiple limitations, including the inability to inactivate genes that would prevent the production of elementary bodies. Many genes are also *Chlamydia*-specific genes, and chlamydial genomes have undergone extensive reductive evolution, so functions often cannot be inferred from homologs in other organisms. Conditional mutants have been used to study essential genes of many microorganisms, so we screened a library of 4,184 ethyl methanesulfonate-mutagenized *Chlamydia trachomatis* isolates for temperature-sensitive (TS) mutants that developed normally at physiological temperature (37°C) but not at nonphysiological temperatures. Heat-sensitive TS mutants were identified at a high frequency, while cold-sensitive mutants were less common. Twelve TS mutants were mapped using a novel markerless recombination approach, PCR, and genome sequencing. TS alleles of genes that play essential roles in other bacteria and chlamydia-specific open reading frames (ORFs) of unknown function were identified. Temperature-shift assays determined that phenotypes of the mutants manifested at distinct points in the developmental cycle. Genome sequencing of a larger population of TS mutants also revealed that the screen had not reached saturation. In summary, we describe the first approach for studying essential chlamydial genes and broadly applicable strategies for genetic mapping in *Chlamydia* spp. and mutants that both define checkpoints and provide insights into the biology of the chlamydial developmental cycle.

IMPORTANCE

Study of the pathogenesis of *Chlamydia* spp. has historically been hampered by a lack of genetic tools. Although there has been recent progress in chlamydial genetics, the existing approaches have limitations for the study of the genes that mediate growth of these organisms in cell culture. We used a genetic screen to identify conditional *Chlamydia* mutants and then mapped these alleles using a broadly applicable recombination strategy. Phenotypes of the mutants provide fundamental insights into unexplored areas of chlamydial pathogenesis and intracellular biology. Finally, the reagents and approaches we describe are powerful resources for the investigation of these organisms.

Chlamydiae are obligate intracellular pathogens that share a characteristic biphasic developmental cycle where these organisms alternate between extracellular, infectious elementary body (EB) and intracellular, replicative reticulate body (RB) forms (1). Multiple species of the family *Chlamydiaceae*, including *Chlamydia trachomatis*, *C. pneumoniae*, and *C. psittaci*, are common or incidental pathogens of humans (2). Despite their medical importance, a lack of tools for genetic manipulation of these organisms was a major impediment in chlamydial research until recently (3, 4).

Only ~60% of *Chlamydiaceae* genes share significant homology with genes of other organisms, and many of these conserved genes mediate essential processes in other bacteria (5). Ancient divergence of *Chlamydia* from other bacterial phyla also allowed high proportions of *Chlamydia*-specific open reading frames (cORFs) to arise in an otherwise-conserved genome (6). System-level searches for essential *Chlamydia* genes have not been reported, but results of three recent studies provide data similar to data that have been used in genetic footprinting analyses for identification of essential genes in other microorganisms (7, 8). Approximately 1.3% of the mutations identified in predicted coding ORFs of a population of 934 randomly selected ethyl methanesulfonate (EMS)-mutagenized *C. trachomatis* isolates were nonsense mutations (9). In contrast, when nonessential *C. trachomatis* (10) and *C. muridarum* (11) genes were sequenced in similarly mutagenized libraries, ~5% of the mutations led to the formation of nonsense codons, consistent with the mutational spectrum of EMS-induced mutations in *Escherichia coli* (12). Overall, these observations suggest that a significant proportion of *C. trachomatis* genes are essential in cell culture.

Tools for genetic modification of *Chlamydia* spp., including

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mutagenesis coupled with forward and reverse genetic analyses (10, 11, 13), intraspecies and interspecies lateral gene transfer (LGT) (14–16), transformation (17), targeted disruption of genes using type II introns (18, 19), and deletion of genes by allelic exchange (20), have been reported, but all have limitations for the study of genes that impact chlamydial fitness in cell culture (3, 21). Another group attempted to identify the lethal temperature-sensitive (TS) allele in a *C. abortus* mutant isolated from a chemically mutagenized library by comparing the genome of that mutant to that of its parent (22, 23). Twenty-two mutations were identified, but the TS phenotype was not linked to any specific mutation (23).

Here we tested the possibility of using conditional mutants to identify genes that mediate *C. trachomatis* survival in cell culture. We describe straightforward approaches for isolating conditional mutants and broadly applicable methods for mapping detrimental *Chlamydia* alleles in natural chlamydial variants and mutants identified in genetic screens. Finally, we identify multiple genes that mediate growth of these organisms in cell culture.

MATERIALS AND METHODS

Cell lines and strains. McCoy and HeLa 229 (HeLa) cells were obtained from the American Type Culture Collection and were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) (HyClone) supplemented with nonessential amino acids, HEPES, and 10% fetal bovine serum (Atlanta Biologicals) (DMEM-10). *C. trachomatis* L2-434/Bu (L2) was transformed with the green fluorescent protein (GFP)-encoding plasmid pGFP::SW2 as described previously (17). The resulting strain (L2-GFP) was plaque purified (24) twice prior to being mutagenized for construction of libraries.

Mutant library construction. McCoy cell monolayers were infected with L2-GFP at a multiplicity of infection (MOI) of 2 by rocking as described previously (25). *Chlamydia* cells were mutagenized by adding 10 ml of ethyl methanesulfonate (EMS) (4 mg/ml) to the infection medium at 18 h postinfection (hpi), using a method similar to one we described previously (11). Mutagenized EBs were harvested from the cells by mechanical disruption at 34 hpi. Mutants were isolated from EB stocks by plaque cloning and were arrayed in cell culture plates. Plaques were expanded in McCoy cells for 70 h. Expanded library plates were stored at −80°C.

Temperature sensitivity screen. HeLa cells were infected with library isolates by centrifugation followed by rocking at 37°C for 30 min. Replicate plates were incubated at 32°C, 37°C, or 40°C. Monolayers were fixed at 34 hpi (for 37°C or 40°C plates) or at 58 hpi (for 32°C plates) with 3.7% formaldehyde. Chlamydial inclusions were imaged at ×4 magnification using an EVOS fluorescence microscope and counted using a custom macro in FIJI (26). Ratios of inclusion-forming units of individual library isolates at permissive temperature versus nonpermissive temperature were then calculated. Isolates that had fold change values of ≥15 were rescreened to confirm their phenotypes. Mutants were plaque cloned twice and expanded in flasks. EBs were released from host cells by bead agitation in sucrose-phosphate-glutamic acid buffer (SPG), and host cell debris was removed by centrifugation at 500 × *g* for 20 min before storage at −80°C. Selected mutants (see Fig. 3) were purified over 30% MD-76R as described by Rajaram et al. (11).

Temperature shift assays. HeLa cells were infected at an MOI of 0.1 as described above. For HS mutants, experimental plates were incubated at 40°C and then moved to 37°C as indicated. Control plates were incubated at 37°C. Cold-sensitive (CS) mutants were incubated at 37°C and then moved to 32°C at the times indicated. The infected cells were fixed at 34 hpi (37°C and 40°C) or 58 hpi (32°C) and labeled with a chlamydial lipopolysaccharide (LPS)-specific monoclonal antibody (EVI-H1) and a secondary fluorophore-conjugated antibody. Inclusions were imaged at ×10 magnification on an EVOS fluorescence microscope (Thermo Scientific). Inclusion cross-sectional areas were measured in CellProfiler (27) using a modified pipeline (28). Mutants were sorted into developmental cycle classes based on the first time interval during which their mean normalized inclusion area was ≤80% of that measured for L2-GFP. Equal adjustments to brightness and contrast of images were made in Adobe Photoshop CS6.

Sequencing library construction and whole-genome sequencing. Genomes were sequenced using two strategies. For some isolates, genomic DNA from 30% MD-76R-purified and DNase-treated EBs (29) was treated with NEBNext double-stranded DNA (dsDNA) Fragmentase (NEB) to generate dsDNA for multiplex Illumina sequencing according to the protocols of the manufacturer (Illumina). A TruSeq Nano DNA sample preparation kit was used to prepare DNA sequencing libraries. Samples were multiplexed using TruSeq single-index sequencing primers, and paired-end 100-bp sequencing was performed at the Tufts University Genomics Core Facility in Boston, MA, on an Illumina HiSeq 2500 system. For other isolates, whole-genome amplification was performed on crude EB preparations using a REPLI-g kit (Qiagen) as described in reference 30, excluding the immunomagnetic cell separation step. These libraries were prepared using a NexteraXT DNA library preparation kit according to the instructions of the manufacturer (lllumina). Samples were multiplexed using a NexteraXT dual-index primer kit, and single-end 100-bp sequencing was performed at the Oregon State University Center for Genomic Research and Biocomputing Core Sequencing Facility (Corvallis, OR) on an Illumina HiSeq 2000 system.

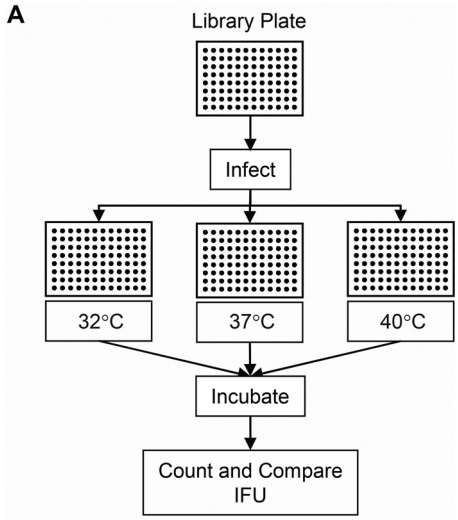
Genome assembly and sequence analysis. Sequence data generated from the TruSeq Nano library were analyzed as described previously (11). Assembly of genome sequences for the NexteraXT library was performed using an alternate workflow method (30) with the additional use of *ad hoc* Python scripts and the Geneious sequence manipulation software suite (version 7) (31) (see Tables S1 and S2 in the supplemental material). Ambiguous sequences from the data set and mutation calls with low-quality scores were resolved by Sanger sequencing. PCR primers are listed in Table S3.

Markerless recombination. HeLa cells were coinfected with pairs of HS or CS mutants with each parent at an MOI of 2, incubated at 37°C, and then harvested at 34 hpi by freeze-thaw in SPG and bead agitation. The harvests were passaged in a blind manner in HeLa cells grown at a nonpermissive temperature and were harvested at 34 hpi, after which the passage and harvest steps were repeated. Recombinants were isolated by limiting dilution, rescreened to confirm temperature resistance, subjected to plaque cloning, and expanded. Genomic DNA was isolated by alkaline lysis. Sanger sequencing was used to determine which single nucleotide polymorphisms (SNPs) present in the parents were retained in the progeny (see Table S2 in the supplemental material).

Statistics. Fold changes of mutant phenotypes at permissive versus nonpermissive temperatures were normalized to fold changes of L2-GFP phenotypes at the same temperatures. Data were subjected to log transformation and analyzed by one-way analysis of variance (ANOVA) with a Dunnett's posttest to detect differences where the P value was <0.05 (GraphPad Prism version 6.0).

RESULTS

Screen for *C. trachomatis* TS mutants. Parallel screens were developed for cold-sensitive (CS) and heat-sensitive (HS) *Chlamydia* TS mutants (Fig. 1A). L2-GFP was mutagenized with EMS, and 4,184 mutant plaques were expanded in McCoy cells. Equal volumes of each isolate were used to infect parallel wells of HeLa cells in three plates, which were then incubated at the permissive (37°C) or nonpermissive (32°C or 40°C) temperature. Approximately 3% of the library isolates were HS in the primary screen, which we defined as isolates that formed 15-fold or greater ratios of GFP-positive (GFP^+) inclusions at 37°C compared to 40°C (Fig. 1B). Five CS isolates which formed 15-fold or greater ratios of inclusions at 37°C compared to 32°C were also identified. To



B

Screen	CS	HS
Primary	5	169
Secondary	2	26

FIG 1 Temperature-sensitive (TS) screen. (A) Corresponding wells of three separate plates of HeLa cells were infected with equal inoculums of isolates from an EMS-mutagenized L2-GFP library, and then the plates were incubated at different temperatures. Inclusions were counted at 34 hpi (37°C and 40°C plates) or 58 hpi (32°C plates). IFU, inclusion-forming units. (B) Numbers of temperature-sensitive isolates observed in the primary and secondary screens, where temperature sensitivity was defined using different stringency thresholds.

confirm phenotypes of the mutants identified in the primary screen, we next performed a more stringent secondary screen where we additionally controlled for differences in the ability of L2-GFP to form inclusions at different temperatures. For the HS mutants, we compared the ratios of inclusions that each of the mutants formed at 37°C compared to 40°C to the ratio of the inclusions that L2-GFP formed at the same two temperatures. Similarly, we compared the ratio of inclusions that the CS mutants formed at 37°C versus 32°C to the ratio of inclusions that L2-GFP formed at the same two temperatures. This allowed us to focus subsequent efforts on 26 HS and 2 CS isolates that met the more stringent criteria in that they formed ≥15-fold ratios of inclusions at the permissive compared to nonpermissive temperature after normalization to L2-GFP (Fig. 1B). Four isolates that were both CS and HS but which did not meet the stringency threshold in the secondary screen were also characterized.

TS mutants are phenotypically diverse. We next performed temporal heat shift assays to test if the TS isolates had similar phenotypes. These assays were performed by shifting infections between permissive and nonpermissive temperatures at various intervals postinfection (Fig. 2A). Infections with the HS mutants were initially incubated at 40°C and then were shifted to 37°C. In contrast, CS mutants were initially incubated at 37°C and then were shifted to 32°C. In both cases, the sizes of the inclusions formed by the mutants in these assays were compared to those seen with L2-GFP grown at the same temperature. Unique defects of the TS mutants became apparent during different temperature shift intervals, allowing them to be sorted into different temporal classes (Fig. 2A and Table 1). We defined these intervals by identifying the earliest temperature shift time when the cross-sectional area of the mutant inclusions fell below 80% of the area of L2-GFP inclusions grown under the same conditions. For example, the cross-sectional area of HS1 inclusions decreased between 12 and 18 hpi when this isolate was incubated at 40°C (Fig. 2B). In contrast, the cross-sectional areas of L2-GFP, and of a recombinant strain that we produced by crossing HS1 and HS13 (HS1 × HS13 [discussed below]), increased during the same interval (Fig. 2B). These assays sorted the mutants into phenotypic classes corresponding to the time during which their TS phenotypes were first detected: prior to 12 hpi (early), 12 to 18 hpi (early-mid), 18 to 24 hpi (mid-late), or over 24 hpi (late) (Table 1). Notably, early-mid and mid-late TS mutants were more abundant than late mutants, consistent with the patterns of *de novo* gene expression in *C. trachomatis* (32).

Next, we performed one-step growth curve analyses to compare the burst sizes of representative TS mutants, selected from the early, early-mid, and mid-late temperature shift classes, at both their permissive and nonpermissive temperatures. The major purpose of this was to quantify how the TS phenotypes impacted the ability of the various mutants to produce infectious EBs. Most of the mutants had burst sizes similar to those seen with L2-GFP at 37°C (Fig. 3A) but produced substantially fewer EBs at the restrictive temperatures (Fig. 3B) than did L2-GFP. The burst sizes of other TS isolates whose phenotypes were eventually mapped to specific alleles, discussed below, were also determined (see Fig. S1 in the supplemental material). Overall, the results of these experiments indicated that most of the TS mutants had minor phenotypes or no phenotype at 37°C but produced fewer infectious progeny at nonpermissive temperatures.

Mutant genotyping. Genome sequencing determined that the TS mutants contained an average of 6 mutations in total (range, 1 to 11), with an average of 4 nonsynonymous mutations in predicted coding ORFs (see Table S1 in the supplemental material). Nonsynonymous mutations were detected in the same gene or ORF in different mutants in 22 cases (2 to 4 occurrences). However, almost all of those mutations were unique, indicating that our screen had not reached saturation. Only two nonsynonymous mutations were identified in genes known to encode central components of heat shock, cold shock, or stress responses in other bacteria ($DksA^{E61K}$ and $ObgE^{G92E}$), and no mutations were identified in *Chlamydia* heat shock genes *groEL*, *groES*, and *dnaK* (33). We could not specifically link *dksA* or *obgE* to temperature sensitivity because the TS mutants that had these alleles also had other mutations (see Table S1). These results showed that the TS phenotypes of most of the mutants were not linked to canonical stress response pathways.

Mapping TS alleles. All but one (HS17; $RpsH^{A119V}$) of the TS mutants contained multiple mutations, which prevented us from linking specific mutations in these mutants to their phenotypes. We next tested if it was possible to produce recombinants of TS mutants by selecting against the parents using the nonpermissive temperature. HS1, HS13, and HS23 were selected for proof-of-principle experiments because these mutants had strong phenotypes and multiple mutations (10, 6, and 4, respectively) for monitoring potential recombination events. Pairs of TS parents were coinfected, and recombinant progeny were selected for by growth at the nonpermissive temperature (40°C). Isolates from each cross

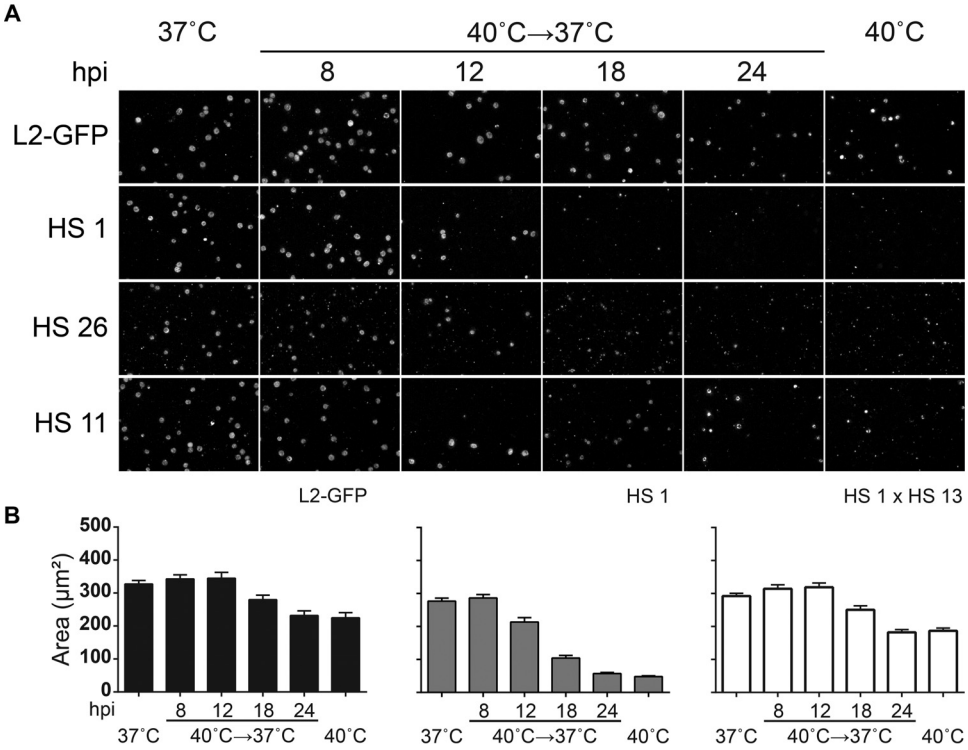


FIG 2 Phenotypes of the TS mutants manifest at different points in the developmental cycle. (A) HeLa cells were infected with L2-GFP or TS mutants and were then incubated at 37°C or 40°C. Some infected cells initially incubated at 40°C were shifted to 37°C at various times postinfection. In all cases, the inclusions were fixed and stained with anti-LPS antibody at 34 hpi. Representative images of early-, early-mid-, and mid-late-stage mutants are shown. Note that the average size of HS1 (early) inclusions decreased dramatically between the 12-h and 18-h temperature shifts, whereas the average size of HS11 (mid-late) inclusions decreased dramatically between the 18-h and 24-h shifts. (B) Comparison of the cross-sectional areas of L2-GFP, HS1, and HS1 × HS13 (a recombinant generated from crossing HS1 and HS13) at 34 hpi following different temperature shift scenarios. Note that the cross-sectional areas of L2-GFP, HS1, and HS1 × HS13 inclusions were similar at 34 hpi when the infections were shifted from 40°C to 37°C at 8 hpi and at 12 hpi but differed significantly when the temperature shift was performed at 18 h postinfection or later.

were purified by limiting dilution, plaque cloned, and confirmed to be temperature resistant (Fig. 4).

Targeted sequencing revealed that the isolates from both the HS1 × HS13 and HS1 × HS23 crosses contained all of the mutations present in HS1, with the exception of the allele encoding GltX^{Q487*}. This result was confirmed by genome sequencing of an isolate from the HS1 × HS13 cross (Fig. 4B; see also Table S2 in the supplemental material). Inclusion morphology and infectious progeny production of the HS1 × HS13 recombinant were indistinguishable from the results seen with L2-GFP and significantly differed from the results seen with both parent strains (Fig. 4C and D). Since only the HS1 *gltX* allele segregated in this cross, it could not be determined if this occurred via genetic reversion or recombination. However, outgrowth of a population passaged at 40°C was observed only in the wells containing both HS1 and HS13 or HS1 and HS23 and not in control wells with an equal inoculum of HS1 EBs (data not shown). Temperature-resistant progeny were also not observed when 1.4×10^9 HS1 EBs were passaged in a blind manner 5 times in HeLa cells at the nonpermissive temperature (data not shown).

TABLE 1 Phenotypic sorting of mutants by cross-sectional inclusion area[a]

Mutant category	No. of isolates			
	Early (0–12 hpi)	Early-mid (12–18 hpi)	Mid-late (18–24 hpi)	Late (>24 hpi)
HS	12	14	3	0
CS	4	0	0	2

[a] Dual HS/CS mutants were counted in both the HS and CS totals.

Twelve TS isolates were crossed and mapped using PCR, targeted Sanger sequencing, and/or genome sequencing. Crosses with multiple parents were required in some cases. The TS alleles were mapped to a single mutant allele in 10 of the 12 strains and were mapped to two closely linked mutant alleles in the other two strains (Table 2; see also Table S2 in the supplemental material). Many crosses produced mosaics, which we defined as strains that contained at least two mutations from each of the parental strains, in contrast to isogenic strains that differed from one of the parents by only a single mutation (Fig. 4A). This confirmed that recombination, and not reversion, was the predominant mechanism involved in generation of temperature-resistant isolates from temperature-sensitive parents. When isogenic progeny could not be isolated, multiple mosaic progeny strains were used to deduce the TS allele through phenotypic linkage as described previously (13).

Four of the TS alleles corresponded to genes whose homologs mediate essential processes in other bacteria, including DNA replication (*dnaE*), tRNA aminoacylation (*gltX*), proton gradient generation (*atpB*), and translation (*rpsH*). Another TS allele

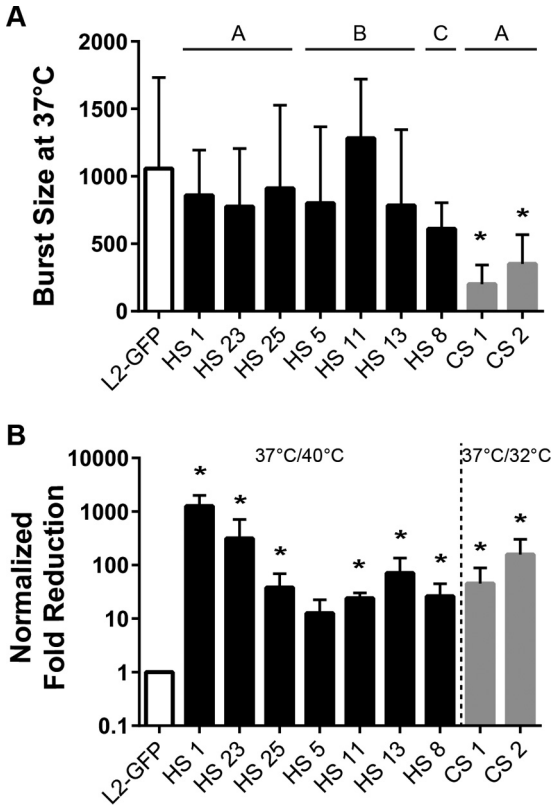


FIG 3 TS mutants produce few infectious EBs at nonpermissive temperatures. (A) Burst sizes were calculated by comparing the number of inclusions TS isolates formed in primary infections to the number formed in secondary infections performed with harvests from the primary infections. Most of the mutants had burst sizes similar to those seen with L2-GFP at 37°C, although the burst sizes of two of the CS mutants were significantly reduced. The mutants are grouped by the temporal classes identified as described for Fig. 2 (A, early; B, early-mid; C, mid-late). (B) Comparison of levels of EB production of the TS mutants at permissive and nonpermissive temperatures. The numbers of progeny produced by HS mutants at 37°C and 40°C were compared, and the numbers of progeny of CS mutants were compared at 37°C and 32°C. This ratio was then compared to the ratio of inclusions formed by L2-GFP at the same two temperatures to determine the fold reduction. The experiments were performed three times in triplicate, and error bars show standard deviations. Mutants with results that differed from those determined for L2-GFP ($P < 0.05$) were identified by one-way ANOVA followed by Dunnett's *post hoc* test and are indicated by asterisks.

mapped to *fabI*, which encodes the enoyl-acyl carrier protein for type II fatty acid biosynthesis, which is essential for cellular replication in *Chlamydia* spp. (34). Finally, 4 alleles mapped to cORFs (*ctl0322*, *ctl0456*, *ctl0597*, and *ctl0681*).

DISCUSSION

TS mutants can provide insights into poorly explored areas of chlamydial biology that cannot be interrogated using existing tools. For example, HS1 contains a nonsense mutation that truncates glutamyl-tRNA synthetase (GltX), which is predicted to aminoacylate both $tRNA^{Glu}$ and $tRNA^{Gln}$ in *Chlamydia* spp. (35). Interestingly, *de novo* translation of some immediate-early proteins (36–38) precedes *de novo* transcription of tRNA, tRNA modification enzymes, and aminoacyl transferases in *C. trachomatis*, including *gltX*, which is predicted to be transcribed at 8 hpi in *C. trachomatis* serovar D (32). Transfer RNAs are abundant in *C. trachomatis* EBs (54), although the aminoacylation status of these is unknown. Proteomic studies (40–42) have also identified aminoacyl-tRNA synthetases in EBs. Since growth of HS1 was rescued when this mutant was shifted from 40°C to 37°C as late as 18 hpi (Fig. 3B), our results suggest that EBs may contain pools of aminoacylated-tRNAs or that tRNAs and functional GltX are preloaded into EBs (Fig. 3B). In future work, we hope to test this hypothesis by determining if EBs contain aminoacylated-$tRNA^{Glu}$ and $tRNA^{Gln}$ using denaturing acid gel electrophoresis and Northern blot analysis and methods similar to those described by Jester et al. (43).

Chlamydia trachomatis encodes a truncated partial tricarboxylic acid (TCA) cycle that cannot use pyruvate as an initial substrate (9, 44). Despite this, *Chlamydia trachomatis* can import the TCA intermediate 2-oxoglutarate (45). Two CS mutants (CS1 and CS3) had nonsense mutations in the putative dicarboxylate transporter CTL0456 (SodTi), which is predicted to import 2-oxoglutarate to feed the TCA cycle (46). Both nonsense mutations truncate the protein before the residues required by the *Vibrio cholerae* SodTi homolog to contact dicarboxylates (47), suggesting that the truncated SodTi in CS1 and CS3 is nonfunctional. The TS allele in HS20 also mapped to *ctl0456*. The $CTL0456^{G145R}$ missense mutation in HS20 is proximal to the first Ser-Asn-Thr motif that would be predicted to contact the dicarboxylate ligand. *C. trachomatis* encodes a putative glutamate transporter, GltT, from which 2-oxoglutarate could be generated from hydrolysis of glutamate by glutamate dehydrogenase (46). However, a GltT nonsense mutant is viable at 37°C (9). These observations suggest that 2-oxoglutarate acquisition is essential in *C. trachomatis* and that redundant pathways for import of this substrate are available at physiological temperatures. Comparing uptake of isotopically labeled 2-oxogluterate and glutamate in TCA mutants using approaches pioneered by the McClarty group (48) could elucidate essential *Chlamydia* TCA cycle substrates.

Some of the TS mutants provide insight into potential functions of cORFs. For example, Grieshaber et al. recently reported that the noncoding RNA (ncRNA) IhtA interferes with the translation of the chlamydial histone-like protein HctA and CTL0322 (49). HctA functions similarly to a eukaryotic histone and mediates condensation of chromatin during the RB-to-EB transition (50–52). Like HctA, CTL0322 is predicted to be a small, highly basic protein, and the *C. trachomatis* serovar D homolog of CTL0322 is nucleotropic in Hep2 cells (53). Greishaber proposed that, similarly to HctA, CTL0322 might bind DNA and mediate the RB-to-EB transition (49). Supporting this hypothesis, HS11, which contains $CTL0322^{M51I}$, has a TS defect that initiates between 18 and 24 hpi, corresponding to the beginning of the RB-to-EB transition in these strains of *C. trachomatis*.

Although our results suggest that the TS approach can identify essential *C. trachomatis* genes, some limitations must be considered. Phenotypes of some of the TS alleles may not be directly linked to thermal inactivation of their encoded proteins. For example, we suspect that the nonsense alleles of *ctl0456* in CS1 and CS3 are nulls. Since many TS mutations are dominant, it also cannot be immediately differentiated if TS alleles are *cis*-acting or *trans*-acting. The TS alleles mapped in this study are not in known ncRNA regions (54–56), and missense mutations usually do not cause polar effects. Another limitation is that the phenotypes of the TS mutants need to be compared to those of the correspond-

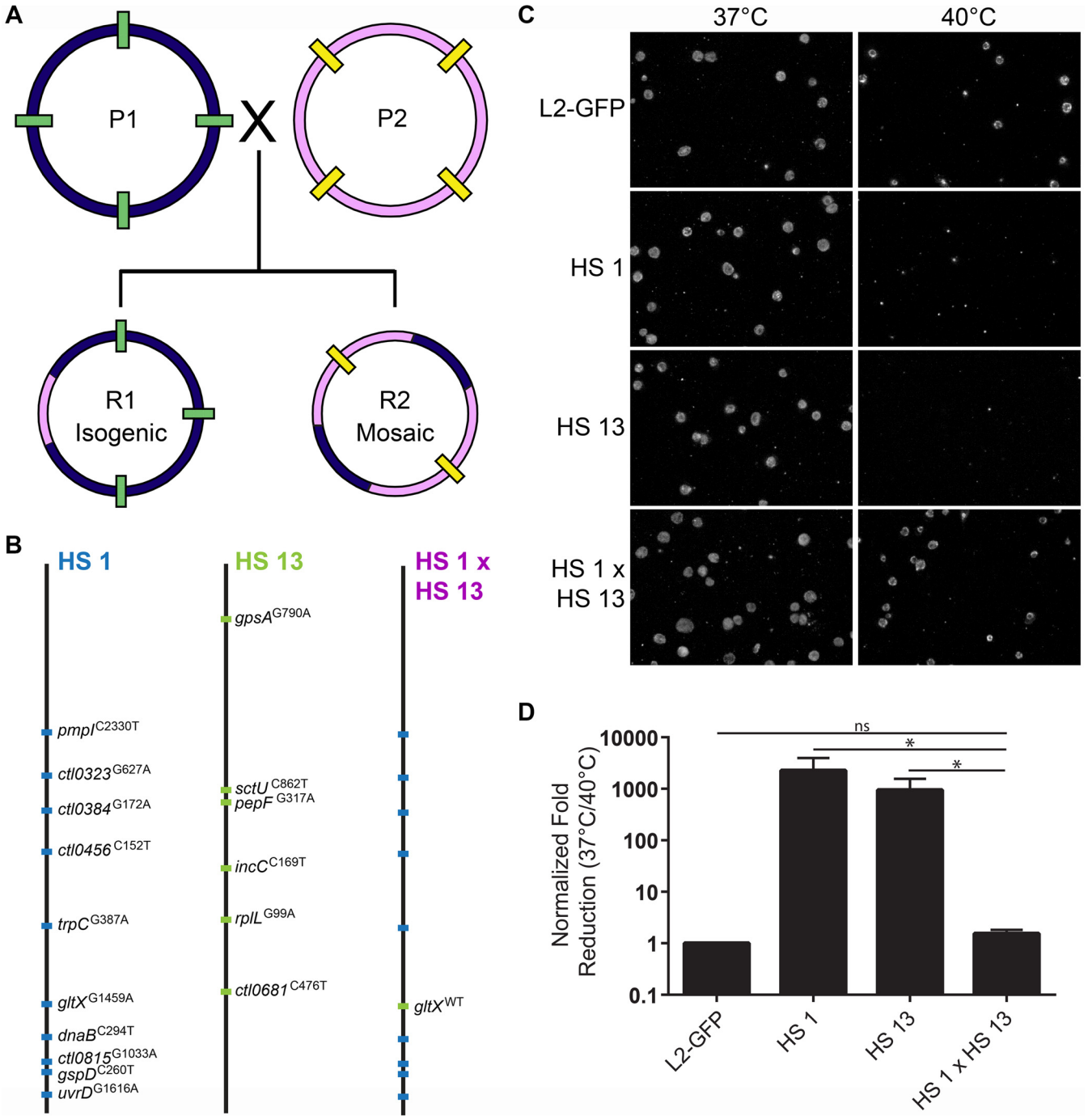


FIG 4 Markerless recombination mapping of TS alleles. (A) Recombinants were generated by coinfecting HeLa cells with two different HS or CS parents and selecting for temperature-resistant progeny at 40°C or 32°C as appropriate. Isogenic and mosaic recombinants resulted. (B) Alleles that differed in the parents of a representative cross, indicated by colored rectangles, were sequenced. (C) Inclusion morphologies of L2-GFP, HS1, HS13, and a recombinant isolate derived from crossing HS1 and HS13 (HS1 × HS13) at 37°C and 40°C, analyzed at 34 hpi. Inclusions were labeled with an anti-LPS antibody. (D) EB production of HS1, HS13, and HS1 × HS13 at 37°C versus 40°C compared to that seen with L2-GFP. The graph shows means of results of 3 experiments performed in triplicate, and the error bars indicate standard deviations. The HS strains produced significantly fewer EB at 40°C than at 37°C than did L2-GFP or recombinant HS1 × HS13 (*, $P < 0.05$), whereas the levels of EB production seen with HS1 × HS13 and L2-GFP did not differ, as determined by one-way ANOVA followed by Dunnett's *post hoc* test. ns, not significant.

ing non-TS parent at the same temperature because *C. trachomatis* developmental kinetics differ at 32°C, 37°C, and 40°C.

TS alleles also have practical applications in genetic analysis. Markerless recombination using TS alleles circumvents some limitations of antibiotic-driven LGT (14, 15). Only a few endogenous resistance alleles in *Chlamydia* spp. have been described, and use of other resistance transgenes is contraindicated due to concerns about clinical resistance. We identified TS alleles located across

TABLE 2 Summary of mapped temperature-sensitive alleles

Mutant	Gene	Amino acid substitution[d]	Function of gene product	TS class(es)
HS1[a]	*gltX*	Q487*	Glu-tRNA synthetase	Early
HS2[c]	*ctl0609*	R198W	Unknown	Mid-early
	phnP	P127L	Phosphonate metabolism	Mid-early
HS10[c]	*rplF*	G109E	Ribosomal protein	Mid-late
	fmt	D276N	fMet-tRNA formyltransferase	Mid-late
HS11[b]	*ctl0322*	M51I	Unknown	Mid-late
HS13[a]	*ctl0681*	P159L	rRNA processing	Mid-early
HS17	*rpsH*	A119V	Ribosomal protein	Mid-early
HS19[b]	*atpB*	G339D	ATPase	Early
HS20[b]	*ctl0456*	G145R	Dicarboxylate transport	HS, early; CS, late
HS23[a]	*dnaE*	P852S	DNA replication	Early
HS24[a]	*fabI*	D79N	Fatty acid biosynthesis	Early
HS26[a]	*ctl0597*	G69R	t^6A^{37} tRNA modification	Mid-early
CS1[b]	*ctl0456*	W321*	Dicarboxylate transport	Early
CS3[a]	*ctl0456*	W442*	Dicarboxylate transport	HS, early; CS, late

[a] Mapped with an isogenic non-TS recombinant.
[b] Mapped by phenotypic linkage analysis of multiple recombinants.
[c] Recombination efforts have not definitively identified a single TS allele.
[d] An asterisk indicates truncation.

the length of the *C. trachomatis* genome. Thus, fine segregation of alleles is possible with the TS LGT strategy due to an abundance of potential recombination partners. We have also observed that both endogenous antibiotic resistance alleles and resistance transgenes can alter chlamydial growth rates and can yield synthetic phenotypes when combined (data not shown), neither of which is a limitation of our markerless strategy. Another potential application of TS alleles is in *trans*-acting and conditional counterselection. We have observed that some of the TS alleles are toxic when expressed from the chlamydial plasmid at nonpermissive temperature (data not shown). This suggests that these alleles could be harnessed for genetic applications analogous to those employing the lethal *Escherichia coli* sucrose *sacB* gene (57).

Overall, multiple characteristics of TS mutants make them powerful tools for broader study of essential and pleiotropic *Chlamydia* genes. First, since most TS mutations are missense mutations, they are ideal for interrogation of essential genes in operons. Second, we have isolated intragenic suppressors of one of the TS mutants (HS23) by passage at nonpermissive temperature (data not shown). This represents a powerful new tool for studying protein-protein interactions. Finally, our existing collection of TS mutants defines a series of temporal checkpoints in the chlamydial developmental cycle. These mutants could be especially useful for ordering closely linked processes in chlamydial development and could provide a mechanism to better understand the transcriptome and proteome throughout development without the complications of asynchrony.

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